PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P0019-JCY		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/KR00/01173	18 OCTOBER 2000 (18.10.2000)	08 APRIL 2000 (08.04.2000)
Applicant		
YOU, Ji-Chang et al		

This International search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
      ☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).
   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:
      ☒ contained in the international application in written form.
      ☒ filed together with the international application in computer readable form.
      ☐ furnished subsequently to this Authority in written form.
      ☐ furnished subsequently to this Authority in computer readable form.
      ☒ the statement that the subsequenlty furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      ☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.
2. ☐ **Certain claims were found unsearchable** (See Box I).
3. ☐ **Unity of invention is lacking** (See Box II).
4. With regard to the **title**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established by this Authority to read as follows:
5. With regard to the **abstract**,
   ☒ the text is approved as submitted by the applicant.
   ☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.
6. The figure of the drawing to be published with the abstract is Figure No. 3
   ☒ as suggested by the applicant. ☐ None of the figures.
   ☐ because the applicant failed to suggest a figure.
   ☐ because this figure better characterizes the invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/01173

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/03

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC(7) C12N

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched
Korean Patent and application for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
IPN, NPS, PAJ, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YOU JC et al., "HIV nucleocapsid protein. Expression in Escherichia coli, purification, and characterization." , J. Biol. Chem., August 1993 268(22), pages 16519-16527.	1
A	STAIR RK et al., "Recombinant retroviral systems for the analysis of drug resistant HIV.", Nucleic Acids Res., October 1993, 21(20), pages 4836-4842.	1, 2
A	BAUM EZ et al., "beta-Galactosidase containing a human immunodeficiency virus protease cleavage site is cleaved and inactivated by human immunodeficiency virus protease.",Proc Natl Acad Sci U S A, December 1990, 87(24), pages 10023-10027.	2

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 FEBRUARY 2001 (12.02.2001)	14 FEBRUARY 2001 (14.02.2001)
Name and mailing address of the ISA/KR Korean Industrial Property Office	Authorized officer CHOI, Kyu-Whan

WHAT IS CLAIMED IS:

1. *E.coli* JM109(KCCM-10194) cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL1234) containing HIV psi(Ψ) gene, SL1234(SEQ ID NO: 2) and β-galactosidase reporter gene(SEQ ID NO: 1).

2. A method for screening HIV packaging inhibitors which comprises the steps of:

(i) culturing *E.coli* JM109(KCCM-10194) of claim 1;

(ii) treating the said *E.coli* with putative compounds or compositions of HIV inhibitors; and,

(iii) measuring the degree of change in β-galactosidase expression in the culture.

INDICATIONS RELATING TO DEPOSITED MICROORGANISM OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13*bis*)

A. The indications made below relate to the deposited microorganism or other biological material referred to in description
On page 6, lines 8-13.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on additional sheet ☐

Name of depositary institution

Korean Culture Center of Microorganisms(KCCM)

Address of depositary institution (including postal code and country)

Korean Culture Center of Microorganisms(KCCM)
361-221, Yurim B/D, Hongje-1-dong, Seodaemun-gu
Seoul, 120-091, Republic of Korea

Date of deposit	Accession Number
Mar. 31, 2000	KCCM-10194

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information continues on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

A TRANSFORMANT FOR SCREENING OF INHIBITORS FOR HUMAN IMMUNODEFICIENCY VIRUS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a transformant for screening of human immunodeficiency virus("HIV") inhibitors, more particularly, to a transformant cotransformed with a plasmid expressing HIV nucleocapsid protein and a plasmid containing HIV psi(Ψ) nucleotide sequence and β-galactosidase reporter gene, and a method for screening of HIV inhibitors by employing the said transformant.

Background of the Invention

HIV, a pathogen causing the aquired immunodeficiency syndrome("AIDS"), selectively infects crucial immune cells called CD4+ T helper cells and replicates inside the cells. Infection of HIV leads to the lysis of CD4+ T cells resulting from an interaction between viral env glycoprotein and plasma membrane of target cell and a subsequent reproduction of virus particles. Also, the binding of soluble gp120 to CD4+ molecules onto uninfected T cells block interactions of CD4+ T cells with other immune cells. In addition to depleting CD4+ T cells, impaired are function of cytotoxic T cells expressing CD8+, antibody-dependent cytotoxicity, maturation of CD4+ T cells in thymus, interaction between CD4+ cells and class II MHC on antigen presenting cells, and function of macrophages and natural killer cells. Thus, human immune system is gradually deteriorated after HIV infection.

Until now, drugs suppressing HIV replication have been developed, which include reverse transcriptase inhibitors such as AZT(azidothymidine) and

ddI(dideoxyinosine), and protease inhibitors. Recently, the researches to develop DNA vaccines employing nucleotide sequence encoding HIV proteins(see: Hinkula J. et al., Vaccine, 15:874-878, 1997; Calarota et al., Lancet, 351:1320-1325, 1998), and live-attenuated HIV vaccines made by deleting the HIV nef gene are being undertaken(see: Kestler and Jeang, Science, 270:1219-1222, 1995; Chakrabarti et al., Proc. Natl. Acad. Sci., USA, 93:9810-9815, 1996).

Since the said drugs are not able to remove the provirus of HIV of which DNA is inserted into the host immune cell chromosome or not able to selectively remove host immune cells containing the provirus, it cannot be excluded that HIV variants arisen by genetic mutation acquire drug resistance or HIV revertants arisen by recombination of attenuated virus vaccine acquire characteristics of pathogenic HIV(see: Berkhout et al., J. Virol., 73:1138-1145, 1999). Furthermore, it has been found that HIV requires not only CD4+ molecule as the receptor on the surface of host cells, but also coreceptors such as 'T-cell-line-tropic' CXCR4/fusin coreceptor or 'macrophage-tropic' CCR5 coreceptor for its binding and gaining entry of HIV into host cells(see: Feng et al., Science, 272:872-877, 1996). Thus, one approach for drug therapy is to target these coreceptors in an attempt to inhibit binding of virus onto the host cells. Since the normal function of these coreceptors is to bind 'chemokines(chemotactic cytokines)' which plays a role in inflammation reaction, serious side effects may be anticipated(see: Murphy, P.M., Ann. Rev. Immunol., 12:593-633, 1994). In view of above situation, there is a need to develop a novel class of HIV inhibitors which do not affect host immunity or physiological activity relating to receptors, and one approach for such drug therapy is to target HIV specific factors required for virus assembly.

When HIV virus particle is assembled, its genomic RNA is selectively packaged into a virion. It is well known

that a specific interaction between the region of nucleocapsid(NC) protein and the viral packaging sequence(encapsidation signal), psi(Ψ), allows selective packaging of viral genomic RNA. Psi(Ψ), located between long terminal repeat(LTR) of 5'-terminal of genomic RNA and *gag* gene which encodes precursor poly protein(matrix-capsid-nucleocapsid), has 4 stem-loop structures. However, the screening of compounds or compositions which inhibit the specific viral interaction required for packaging is difficult due to the lack of an easy and efficient assay system for such screening.

Under the circumstances, there are strong reasons for exploring and developing a model system which can be used to detect the specific interaction between HIV NC protein and HIV psi(Ψ) sequence as in *in vivo*, for screening of inhibitors against HIV packaging.

Summary of the Invention

The present inventors have made an effort to develop a simple and effective method for detecting the specific interaction between HIV NC protein and HIV psi(Ψ) sequence in vivo to screen HIV packaging inhibitors, thus, prepared transformants cotransformed with a plasmid expressing HIV NC protein and a plasmid containing HIV psi(Ψ) sequence and β-galactosidase reporter gene, and found that HIV inhibitors can be conveniently screened by employing the said transformant on the basis of the expression level change of β-galactosidase.

A primary object of the invention is, therefore, to provide a transformant cotransformed with a plasmid expressing HIV nucleocapsid protein, and a plasmid containing HIV psi(Ψ) sequence and β-galactosidase reporter gene.

The other object of the invention is to provide a method for screening HIV packaging inhibitors employing the

said transformant.

BRIEF DESCRIPTION OF THE DRAWINGS

The above, the other objects and features of the invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1a is a schematic representation of construction strategy of pX1(-ATG).

Figure 1b is a schematic representation of construction strategy of pNH1.

Figure 1c is a schematic representation of construction strategy of pNH1Psi or pNH1rePsi.

Figure 2 is a graph showing the expression of β-galactosidase in *E. coli* JM109 cotransformed with each of pJC1 expressing nucleocapsid protein, or pTrcHisGag expressing Gag protein, or pSE380 as a control plasmid, and pNH1, respectively.

Figure 3a is a graph showing the effect of interaction between HIV nucleocapsid protein or Gag protein and HIV psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in *E. coli* JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1MCS, respectively.

Figure 3b is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and HIV psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in *E. coli* JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1Psi(SL1234), respectively.

Figure 4 is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and various portions of psi(Ψ) sequence on the expression of β-galactosidase in *E. coli* JM109 which is cotransformed with each of pJC1, pTrcHisGag or pSE380 and each of plasmid pNH1Psi(SL1234), pNH1Psi(SL234), pNH1Psi(SL34), pNH1Psi(SL23), or pNH1Psi(SL12) containing psi(Ψ) nucleotide sequence, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The invented transformant employed for screening of HIV packaging inhibitors is prepared by cotransforming a plasmid pJC1 expressing HIV NC protein and a plasmid pNH1Psi(SL1234) containing HIV psi(Ψ) gene and β-galactosidase reporter gene.

The process for preparing the transformant for screening of HIV inhibitors is further illustrated in more detail.

The transformant was prepared by cotransforming with pJC1 expressing HIV NC protein and pNH1Psi(SL1234) containing HIV psi(Ψ) gene and β-galactosidase reporter gene: pX1(-ATG) was constructed by removing ATG from pX1 plasmid which was made by inserting lacZ gene fragment into pSE280. And then, pZ1 was obtained by substituting ampicillin resistance gene of pX1(-ATG) with the AflIII-StuI fragment containing kanamycin resistance gene. pNH1 containing lacZ gene was constructed by inserting HindIII-BspH1 fragment containing rrnB T1T2 terminator from pX1(-ATG) into pZ1. pNH1Psi(SL1234) containing lacZ gene which is flanked by HIV psi(Ψ) nucleotide sequence right before the starting codon was constructed by inserting 4 stem-loop structure-containing psi(Ψ) fragment from pLLIII into pNH1 constructed above(see: Figure 1). And then, NC protein-

expressing pJC1 vector which is constructed by the present inventors(see: Ji Chang You and Charles S. McHenry, J. Biol. Chem., 268:16519-16527, 1993) and pNH1Psi(SL1234) constructed above are cotransformed into *E.coli*. That is, vectors pJC1 and pNH1Psi(SL1234) are cotransformed into *E.coli* by electroporation, followed by selecting antibiotics resistant transformant in medium containing antibiotics. The transformant was named *E.coli*/pNH1Psi(SL1234), and deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea), under an accession(deposition) No. KCCM-10194 on March 31, 2000. The transformant prepared above has a characteristic that β -galactosidase reporter gene expression is down regulated due to the interaction of psi(Ψ) nucleotide sequence with HIV NC protein, thereby it can be used for screening of HIV packaging inhibitors. For instance, the culture of said transformant is treated with putative compounds or compositions of HIV inhibitors, and then the expression level change of β -galactosidase in culture is determined. Therefore, the transformant of the invention can be used for screening of HIV packaging inhibitors which block the binding of HIV NC protein to HIV psi(Ψ) nucleotide sequence.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Construction of pNH1(SL1234)

A plasmid containing β-galactosidase reporter gene(SEQ ID NO: 1) was constructed.

Example 1-1: Construction of pNH1

LacZ gene fragment obtained by PstI (Boeringer Mannheim, Germany) digestion of pUS935(see: Dellagostin, O. A. et al., Microbiology, 141:1785-1792, 1995) was inserted into pSE280(Invitrogen, U.S.A.) to obtain pX1. In order to prevent translation from starting at ATG which is located upstream of the multicloning site of LacZ fragment, upstream ATG was removed by cutting pX1 with NcoI, mung bean exonuclease(Boehringer Mannheim, Germany) treatment, Klenow enzyme treatment to create blunt end and then ligating to make pX1(-ATG). Figure 1a is a schematic representation of construction strategy of pX1(-ATG). pZ1 was made by substituting ampicillin resistance gene of pX1(-ATG) with a kanamycin resistance gene, that is, by ligating the lacZ gene-containing MscI fragment from pX1(-ATG) and Kan^r gene-containing AflIII-StuI fragment from pZerO-2(Invitrogen, U.S.A.). Then, pNH1 was made by inserting rrn T1T2 terminator-containing HindIII-BspHI fragment from pX1(-ATG) into the ScaI site of pZ1. Figure 1b is a schematic representation of construction strategy of pNH1.

Example 1-2: Construction of pNH1Psi(SL1234)

pLLIII(University of Colorado, Health Sciences Center, Charles S. McHenry), a plasmid containing the 5' long terminal repeat(LTR) of HIV-1, was digested with SacI and MseI and then treated with Klenow enzyme to obtain a fragment containing HIV psi(Ψ) nucleotide sequence(named "SL1234", SEQ ID NO: 2) which contains 4 stem-loop structures.

SL1234 was digested with MaeI and then treated with Klenow fragment to obtain SL12(SEQ ID NO: 3). pNH1Psi(SL1234) or pNH1Psi(SL12) was made by inserting SL1234 or two fragments of SL12 into the BstEII site which is located upstream of lacZ gene in pNH1, respectively. Then, pNH1rePsi(SL1234) or pNH1rePsi(SL12) containing HIV psi(Ψ) nucleotide sequence in reverse orientation was made

to be used as a control vector. DNA bands of 221bp for right orientation and 152bp for reverse orientation were identified by 2% agarose gel electrophoresis.

Primers, U2 and L3 pair or U2 and L4 pair, were used for amplification of SL23(SEQ ID NO: 4) or SL234(SEQ ID NO: 5), respectively, and their nucleotide sequences are as follows.

primer U2 (SEQ ID NO: 6)
5'-GGG**GGTGACCTTTAAA**AGCAAGAGGCGAGGG-3'
primer L3 (SEQ ID NO: 7)
5'-GGG**GGTGACC**CTCTCCTTCTAGCCTCCG-3'
primer L4 (SEQ ID NO: 8)
5'-GGG**GGTGACC**GACGCTCTCGCACCCGTCTCT-3'

In order for easier insertion of psi(Ψ) fragment into BstEII site in pNH1, BstEII site(boldface) was inserted into the each primer, and DraI site(boldface) was inserted into the primer U2 to identify orientation. Also, point mutation(underlined), T to G, was introduced into primer L4 to prevent translation from starting in the psi(Ψ) sequence. PCR was performed in a 100 $\mu\ell$ of reaction mixture containing 10mM Tris-HCl, pH 7.5, 1.5mM $MgCl_2$, 50mM KCl, 1mM dNTP, 10μ M of each primer, 1 μg of pLLIII and 2.5 unit of Taq polymerase(Boehringer Mannheim, Germany) with preincubation at 94℃ for 5min, 30 cycles of denaturation at 94℃ for 1min, primer annealing at 60℃ for 2min, and extension at 72℃ for 2min, and then additional extension at 72℃ for 10 min. For SL23 insertion, DNA bands of 213bp for right orientation and 146bp for reversed orientation were identified by 2% agarose gel electrophoresis following digestion of each plasmid with DraI. For SL234 insertion, DNA bands of 234bp for right orientation and 146bp for reversed orientation were identified by 2% agarose gel electrophoresis following digestion of each plasmid with DraI.

SL34 fragment obtained by digestion of PCR product,

SL234, with RsaI and BstEII, was treated with Klenow fragment, and then inserted into the BstEII site of pNH1 to make pNH1Psi(SL34) and pNH1rePsi(SL34) as a control. DNA bands of 40bp for right orientation and 56bp for reversed orientation were identified by 4% agarose gel electrophoresis following double-digestion of each plasmid with BsmAI and XmnI.

In order to use as a negative control, that is, to see whether NC protein or Gag protein can interact with nucleotide sequence which is non-homologous to psi(Ψ), pNH1MCS was made by inserting 75bp MCS(multicloning site) fragment, which has no ATG starting codon, obtained by digestion of pSE280 with MaeI and subsequent Klenow treatment, into the BstEII site of pNH1. DNA band of 229bp was identified by 2% agarose gel electrophoresis following RsaI digestion. Figure 1c is a schematic representation of construction strategy of pNH1Psi or pNH1rePsi.

Example 2: Expression of HIV NC protein or HIV Gag protein

NC protein-expressing plasmid, pJC1, which was made and disclosed by the present inventors(see: Ji Chang You, and Charles S. McHenry, J. Biol. Chem., 268(22):16519-16527, 1993) or Gag protein-expressing plasmid, pTrcHisGag, was transformed into *E.coli* strain JM109(Promega Co., U.S.A.) by electroporation: to obtain competent cells, overnight culture of JM109 started from single colony was inoculated into 200ml of fresh LB medium(Luria-Bertani, Difco, U.S.A.) with 1:100 dilution and cultivated to OD 0.5-0.7, and then cells were centrifuged at 4000 g for 10 min., followed by 4 times of washing process which was resuspending cell pellet in cold 10%(v/v) glycerol and centrifugation at 4000 g for 10 min. After being resuspended in 1ml, 20μl of competent cells were mixed with 1-100 ng of DNA, transferred to 0.2 cm gap electroporation cuvette, and transformed in *E.coli* pulser(Bio-Lab, Heracules, CA, U.S.A.) by applying 2.5kV electric shock. The transformant was incubated overnight

in LB medium containing 100 μg of ampicillin, and then subcultured in fresh LB medium with 1:100 dilution. At the early period of logarithmic phase of growth, 1mM of isopropyl-β -D-thiogalactopyranoside(IPTG, Roche Diagnostics, Germany) was added and the proteins were induced for 3 hours. The liquid culture of transformant was sampled at each hour and centrifuged at 10,000 g for 1 min. The cell pellet was resuspended in 20 μl of gel loading buffer(50mM Tris-HCl, pH 6.8, 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol), and incubated for 5 min at 100℃ to disrupt cell wall. After centrifugation at 10,000 g for 30 min, each supernatant was subject to 15% SDS-PAGE at 120V for 2 hours. The gel was stained in staining solution(0.25% Coomassie Brilliant Blue, 45% methanol and 10% glacial acetic acid) for 20 min and then destained in destaining solution(30% methanol and 10% acetic acid) for 2 hours. Although HIV NC protein(7kD) band did not appear without IPTG induction, the protein band at 7kD position was getting thicker after IPTG induction as time went by. Also, HIV Gag protein(55kD) band appeared at about 55kD position after IPTG induction.

Example 3: Cotransformation and measurement of β -galactosidase expression

In order to measure the effect of interaction between HIV psi(Ψ) nucleotide sequence and HIV NC protein or Gag protein on β -galactosidase expression level, pJC1 and each of pNH1Psi plasmids or pTrcHisGag and each of pNH1Psi plasmids were cotransformed into *E.coli* JM109 by electroporation, respectively. To exclude the possibility of plasmid copy number effect and to confirm the expression of lac repressor, JM109 was cotransformed with pSE380(Invitrogen, U.S.A.) as a control plasmid and each of pNH1Psi plasmids. Each cotransformant was selected on LB agar plate containing 100 μg/㎖ ampicillin and 40 μg/㎖ kanamycin.

To measure the expression of β -galactosidase in the cotransformants, β -galactosidase liquid assay was performed for each cotransformant more than 3 times. Each cotransformant was cultured in LB medium overnight, and the culture broth was inoculated into 5ml of fresh LB medium with 1:10 dilution. At the early stage of logarithmic phase of growth, 1ml aliquot of culture broth was taken and kept on ice, and then incubation was continued for 3 hours to allow expression of NC protein, Gag protein and β -galactosidase after 1mM IPTG was added for induction of β -galactosidase. And then, 1ml aliquot of culture broth was sampled each hour and kept on ice. Cell density was measured at A_{600} with enzyme-linked immunosorbent assay reader(ELISA reader, Dynatech, U.S.A.). For measurement of β -galactosidase activity, 50 μl aliquot of each cotransformant culture broth was mixed with 450 μl aliquot of Z buffer(60mM $Na_2HPO_4 \cdot 7H_2O$, 40mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4 \cdot 7H_2O$, 50mM β -mercaptoethanol). And then, cell wall was disrupted by addition of 20 μl of chloroform and 10 μl of 0.1% SDS followed by stirring for 30 seconds and incubating at 28℃ for 5min. To the cell lysate, 100 μl of o-nitrophenyl-1-thio-β -D-galactopyranoside(ONPG, 4mg/ml, Sigma, U.S.A.) was added and the mixture was incubated at room temperature until yellow color developed. When yellow color was fully developed, the reaction was stopped by adding 200 μl of 1M Na_2CO_3, and the mixture was clarified by centrifugation at 10,000 g for 30 min. Optical density(OD) of supernatant was measured at 420nm and 550nm. The activity(units) of β -galactosidase was quantitated by following equation:

$$\text{Enzyme unit of } \beta\text{-galactosidase} = 1{,}000 \times (OD_{420} - 1.75 \times OD_{550}) / (t \times OD_{600})$$

wherein,

t is reaction time(minutes); and,

1.75 is a constant.

In order to determine if NC protein or Gag protein affect the β-galactosidase expression from the plasmid without psi(Ψ) sequence, *E.coli* JM109 was cotransformed with the precursor plasmid, pNH1(containing lacZ gene, no psi(Ψ) sequence) and pJC1(nucleocapsid), pNH1 and pTrcHisGag(Gag protein), or pNH1 and pSE380(control), respectively, and then, β-galactosidase activity was measured as described above. Figure 2 is a graph showing the expression of β-galactosidase in JM109 cotransformed with each of pJC1 expressing nucleocapsid protein, pTrcHisTag expressing Gag protein, or pSE380 as a control plasmid, and pNH1. As shown in Figure 2, the level of β-galactosidase expression in cotransformant expressing NC protein or Gag protein was not different from that in cotransformant expressing no other protein(pSE380) after 3 hour-induction of β-galactosidase with IPTG, therefore, it was demonstrated that NC protein or Gag protein had no effect on the expression of β-galactosidase in cotransformants.

In order to demonstrate the specificity of the interaction between HIV NC protein and HIV psi(Ψ) sequence or Gag protein and HIV psi(Ψ) sequence, β-galactosidase expression was measured in cotransformants containing each of pSE380, pJC1 or pTrcHisGag and each of pNH1MCS(multiple cloning site, MCS, as a non-homologous sequence to HIV psi(Ψ) nucleotide sequence) or pNH1Psi(SL1234, HIV psi(Ψ) nucleotide sequence), respectively. Figure 3a is a graph showing the effect of interaction between HIV nucleocapsid protein or Gag protein and non-homologous sequence to psi(Ψ) on the expression of β-galactosidase after induction with IPTG in JM109 cotransformed with each of pJC1, pTrcHisTag or pSE380 and pNH1MCS. Figure 3b is a graph showing the effect of specific interaction between HIV nucleocapsid protein or Gag protein and homologous psi(Ψ) sequence on the expression of β-galactosidase after induction with IPTG in JM109 cotransformed with each of pJC1, pTrcHisGag or pSE380 and pNH1Psi(SL1234),

respectively. As shown in Figure 3a, the level of β -galactosidase in cotransformants containing non-homologous sequence(pNH1MCS) to psi(Ψ) and a plasmid expressing NC protein or Gag protein was not different from that in cotransformant containing pHN1MCS and control plasmid, pSE380. Meanwhile, as shown in Figure 3b, when transformed together with pNH1psi(SL1234), the level of β -galactosidase in cotransformant expressing Gag protein was reduced by 15% compared to that in cotransformant containing control vector, pSE380, moreover, cotransformant expressing NC protein showed more than 90% reduction in β -galactosidase expression compared to the control cotransformant.

Therefore, it was demonstrated that β -galactosidase expression in cotransformant was reduced by HIV NC protein in the presence of HIV psi(Ψ) nucleotide sequence and the reduction was caused by a specific interaction between HIV NC protein and HIV psi(Ψ) nucleotide sequence.

In order to identify which portion of psi(Ψ) nucleotide sequence is responsible for the specific interaction with NC protein, each of plasmids containing various portions of stem-loop structures of psi(Ψ) nucleotide sequence were cotransformed with NC- or Gag-expressing vector into *E. coli* JM109, respectively, and then β -galactosidase activity of each cotransformant was measured. Figure 4 is a graph showing the effect of specific interaction between HIV NC protein or Gag protein and portions of psi(Ψ) sequence on the expression of β -galactosidase in *E. coli* JM109 cotransformants. *E. coli* JM109 was cotransformed with each of pJC1, pTrcHisGag, or pSE380 and each of plasmids containing various portion of stem-loop structures of psi(Ψ) nucleotide sequence, pNH1Psi(SL1234), pNH1Psi(SL234), pNH1Psi(SL34), pNH1Psi(SL23), or pNH1Psi(SL12), respectively. In Figure 4, horizontal axis indicates the cotransformants containing each of pJC1, pTrcHisGag, or pSE380 and each of psi(Ψ)

structure-containing plasmids(lane 1 is control plasmid, pNH1; lane 2, a plasmid containing non-homologous sequence, pNH1MCS; lane 3, pNH1Psi(SL1234); lane 4, pNH1Psi(SL234); lane 5, pNH1Psi(SL34); lane 6, pNH1Psi(SL23); and, lane 7, pNH1Psi(SL12)), and vertical axis indicates the activity of β -galactosidase. The results were statistically analyzed by Student's test and * indicates $p<0.05$. As shown in Figure 4, HIV NC protein reduced the expression of lacZ gene more effectively when psi(Ψ) sequence is present in cell than HIV Gag protein did. When pNH1Psi(SL1234) containing full length psi(Ψ) nucleotide sequence and pJC1 were cotransformed into pJM109, the reduction of lacZ expression was most significant, and the degree of reduction was decreased in the order of SL34, SL234, SL23, and SL12. The sequence of SL4 appears to be more important than other stemp-loop structures for the specific interaction of psi(Ψ) sequence with NC protein which resulted in the reduction of lacZ gene expression.

Therefore, these results demonstrated that all of 4 stem-loop structures in psi(Ψ) sequence was required for the most significant reduction of β -galactosidase expression, and sequence of SL4 was the most important portion in psi(Ψ) sequence for NC protein binding. And, the degree of reduction of lacZ gene expression in pNH1Psi(SL234) and pNH1Psi(SL34) which were point-mutated to avoid translation starting from SL4 area was similar to that in pNH1Psi(SL1234) without point mutation. Thus, the transformant was named as *E.coli*/pNH1Psi(SL1234), which showed the most significant reduction of β -galactosidase expression by specific interaction of psi(Ψ) sequence with NC protein. *E.coli*/pNH1Psi(SL1234) was deposited with an international depository authority, the Korean Culture Center of Microorganisms(KCCM, #361-221 Hongje-1-dong, Seodaemun-gu, Seoul, Republic of Korea), under an accession(deposition) No. KCCM-10194 on March 31, 2000. The results obtained from the above Examples were summarized in Table 1 below.

Table 1: Level of β -galactosidase expression depending on kinds of reporter vectors

Reporter Vector		Enzyme units of β -galactosidase after 3-hour induction					
		pSE380 (Control)		pJC1 (expressing NCp7)		pTrcHisGag (expressing Gag)	
pNH1	-	302±19*	100%	295±28.7	98%	298±20.8	96%
pNH1MCS	75bp	296±24.5	100%	234±40.5	80%	296±44.5	100%
pNH1Psi (SL1234)	SL1234	119±22	100%	8±2.2	6.7%	103±23	86.5%
pNH1Psi (SL234)	SL234	308±42.5	100%	87±25.5	28%	140±44	76%
pNH1Psi (SL34)	SL34	85±14	100%	11±2.6	13%	68±12	80%
pNH1Psi (SL23)	SL23	298±55.5	100%	112±14	38%	214±22.5	72%
pNH1rePsi (SL12)	SL12	186±32	100%	107±3.5	58%	158±24	85%
pNH1rePsi (SL1234)	reSL1234	87±21	100%	51±10	59%	85±17	98%
pNH1rePsi (SL234)	reSL234	185±37	100%	78±5	42%	140±30.5	76%
pNH1rePsi (SL34)	reSL34	311±29	100%	186±	60%	307±30	99%
pNH1rePsi (SL23)	reSL23	305±43	100%	131±19	43%	280±18	92%
PNH1Psi (SL12)	reSL12	86±21	100%	64±11	75%	86±19.5	100%

As a result, it is a characteristic of the transformant to be reduced in β -galactosidase reporter gene expression due to the specific interaction between psi(Ψ) nucleotide sequence and HIV NC protein. Therefore, the transformant of the invention can be used for screening of HIV packaging inhibitors which block the binding of HIV NC protein to HIV psi(Ψ) nucleotide sequence, by treating the culture broth of *E.coli* JM109(KCCM-10194) with putative compounds or compositions of HIV inhibitors and measuring the degree of change in β -galactosidase expression.

As clearly illustrated and demonstrated above, the present invention provides a microorganism cotransformed with a gene expressing HIV nucleocapsid protein and a plasmid vector containing HIV Psi(Ψ) gene and β -

galactosidase reporter gene, and a method for screening HIV packaging inhibitors employing the said transformant. The invented method comprising the steps of culturing the said transformant, treating it with putative compounds or compositions of HIV inhibitors, and measuring the degree of change in β -galactosidase expression in the culture, can be practically applied in screening HIV packaging inhibitors by which the interaction between HIV nucleocapsid and HIV psi(Ψ) sequence is blocked.

WHAT IS CLAIMED IS:

1. *E.coli* JM109(KCCM-10194) cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL1234) containing HIV psi(Ψ) gene, SL1234(SEQ ID NO: 2) and β-galactosidase reporter gene(SEQ ID NO: 1).

2. A method for screening HIV packaging inhibitors which comprises the steps of:

(i) culturing *E.coli* JM109(KCCM-10194) of claim 1;

(ii) treating the said *E.coli* with putative compounds or compositions of HIV inhibitors; and,

(iii) measuring the degree of change in β-galactosidase expression in the culture.

AMENDED CLAIMS

[received by the International Bureau on 01 August 2001 (01.08.01); original claims 1and 2 replaced by amended claims1-20(3pages)]

1. A microorganism cotransformed with a plasmid vector containing a gene expressing HIV nucleocapsid protein, and a plasmid vector containing HIV psi(Ψ) gene and β -galactosidase reporter gene.

2. The microorganism of claim 1 wherein the plasmid vector containing a gene expressing HIV nucleocapsid protein is pJC1.

3. The microorganism of claim 1 wherein the HIV psi(Ψ) gene is SL1234(SEQ ID NO: 2).

4. The microorganism of claim 1 wherein the HIV psi(Ψ) gene is SL234(SEQ ID NO: 5).

5. The microorganism of claim 1 wherein the HIV psi(Ψ) gene is SL23(SEQ ID NO: 4).

6. The microorganism of claim 1 wherein the HIV psi(Ψ) gene is SL12(SEQ ID NO: 3).

7. The microorganism of claim 1 wherein the β -galactosidase reporter gene is SEQ ID NO: 1.

8. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(Ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL1234).

9. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(Ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL234).

10. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(Ψ) gene and β -galactosidase reporter gene is pNH1Psi(SL23).

11. The microorganism of claim 1 wherein the plasmid vector containing HIV psi(ψ) gene and β-galactosidase reporter gene is pNH1Psi(SL12).

12. *E.coli* JM109(KCCM-10194) cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL1234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

13. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

14. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL23) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

15. A microorganism cotransformed with a vector pJC1 expressing HIV nucleocapsid protein, and a vector pNH1Psi(SL12) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

16. A microorganism transformed with a vector pNH1Psi(SL1234) containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1).

17. A microorganism wherein both a plasmid vector containing a gene coding for HIV nucleocapsid protein and a plasmid vector containing HIV psi(ψ) gene and β-galactosidase reporter gene(SEQ ID NO: 1) are integrated into a chromosome.

18. A method for screening HIV packaging inhibitors which comprises the steps of:

(i) culturing the cotransformed microorganism of claim 1;

(ii) treating the said cotransformed microorganism with putative compounds or compositions of HIV inhibitors; and,

(iii) measuring the degree of change in β-galactosidase expression in the culture.

19. The method of claim 21 wherein the cotransformed microorganism is *E.coli* JM109(KCCM-10194).

20. Use of the cotransformed microorganism of claim 1 for HTS(High Throughput Screening) of HIV inhibitors.

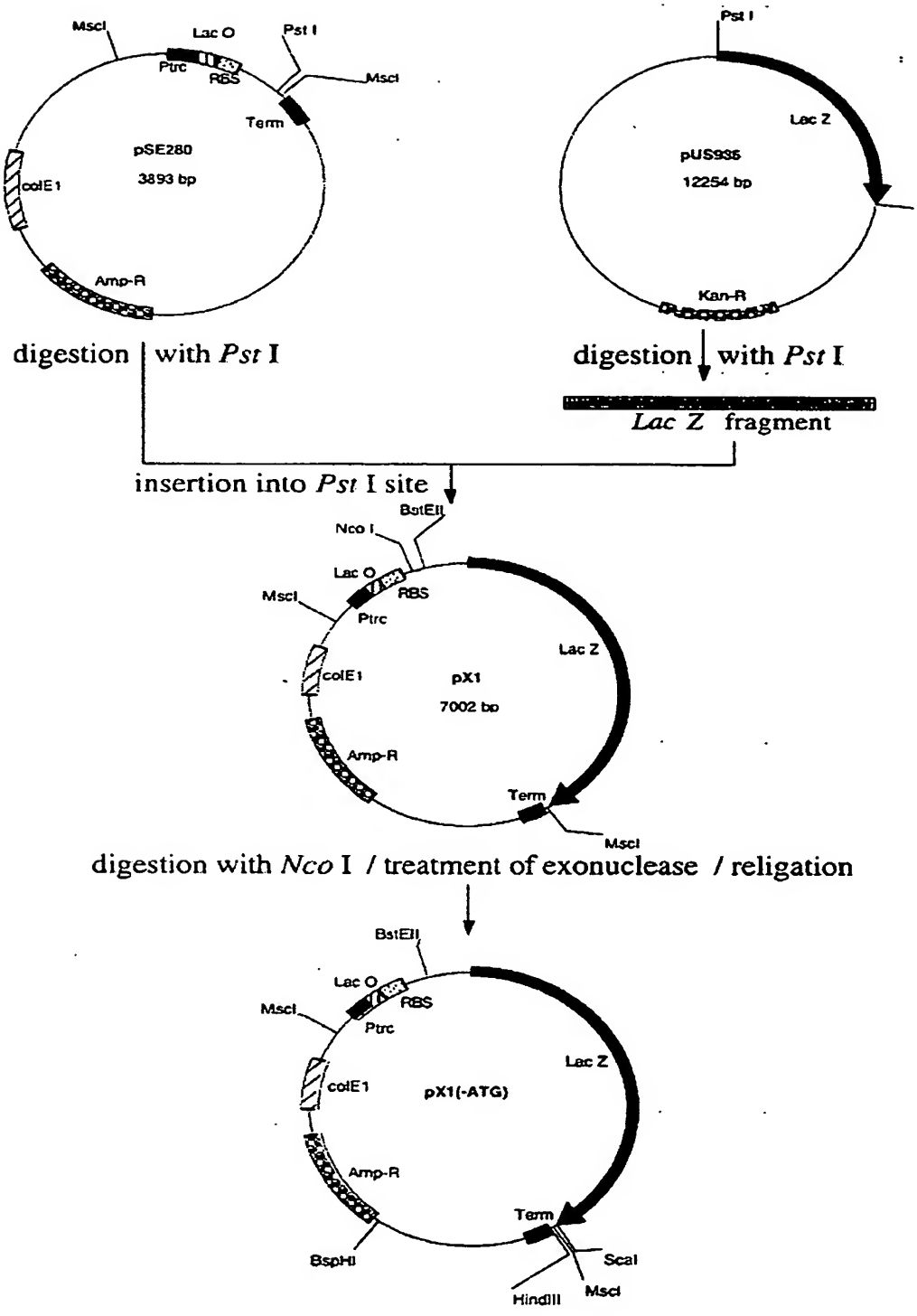
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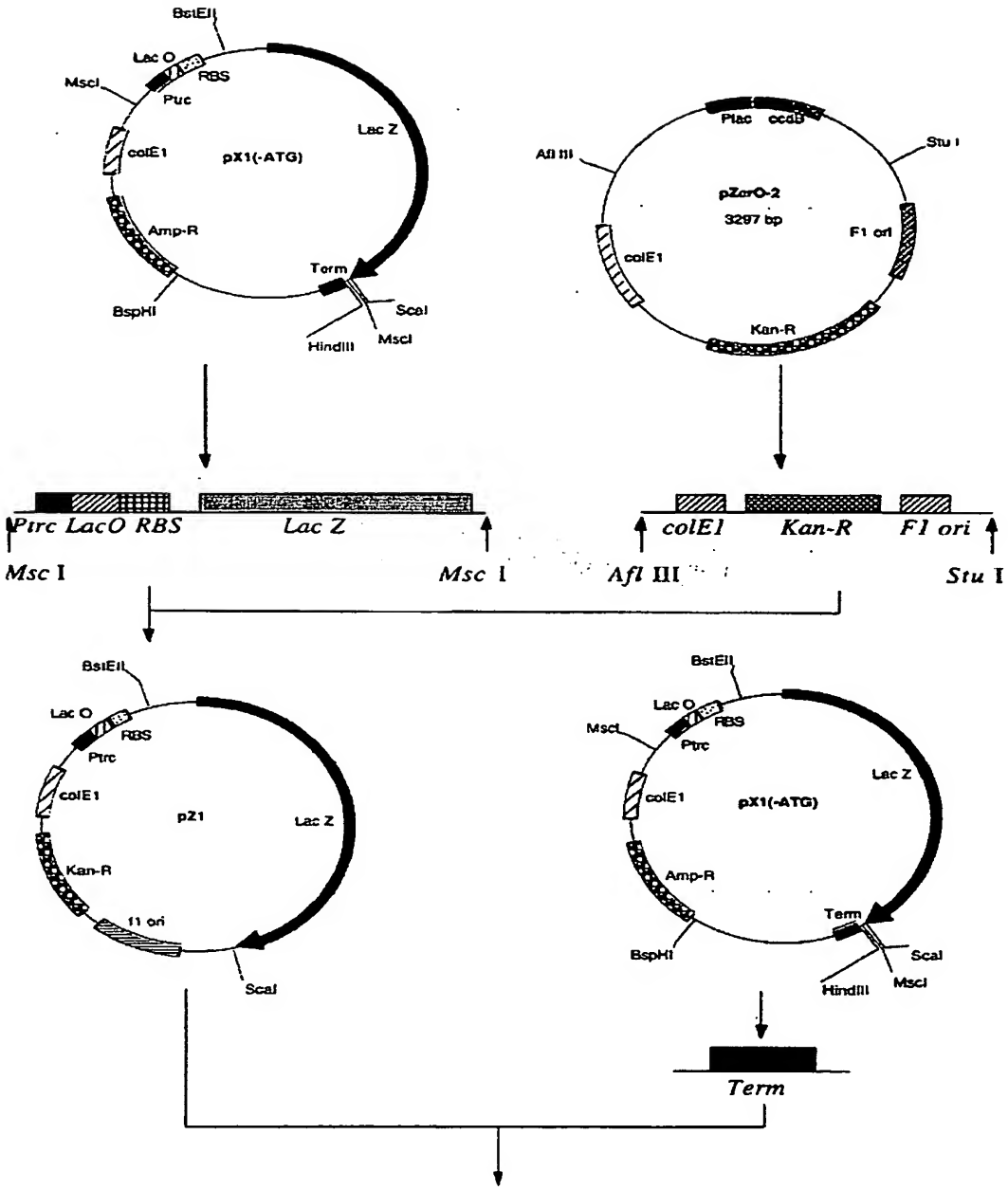
Fig. 1a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AflIII
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Ptrc LacO RBS
Lac Z
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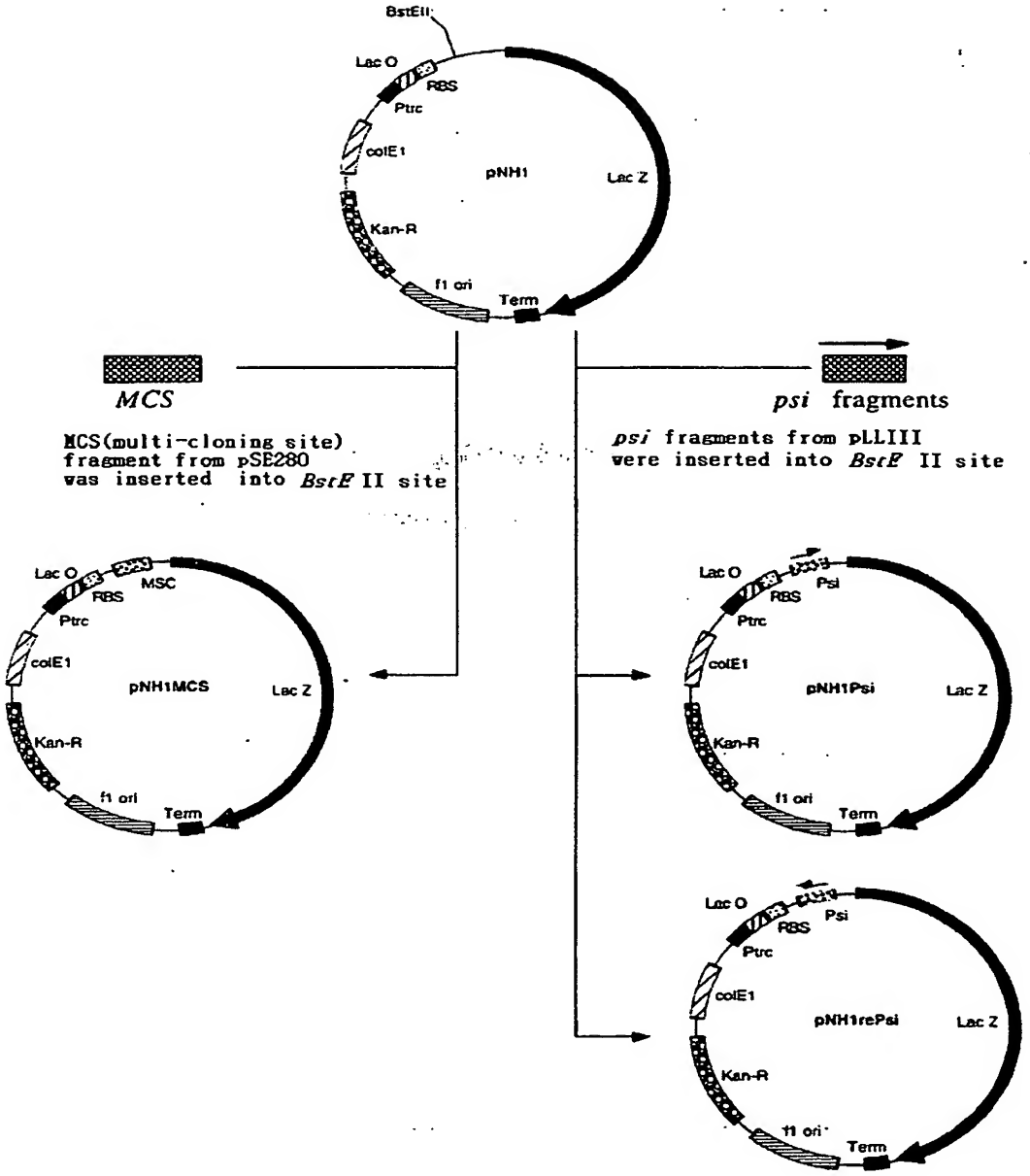
Fig. 1b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was inserted into BstE II site
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were inserted into BstE II site
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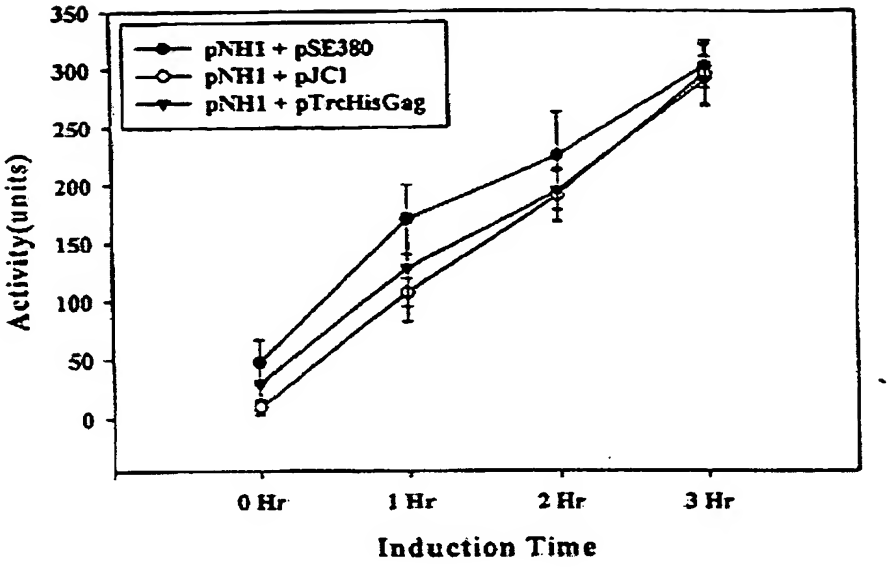
Psi
pNH1Psi
pNH1rePsi

Fig. 1c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pNH1 + pTrcHisGag
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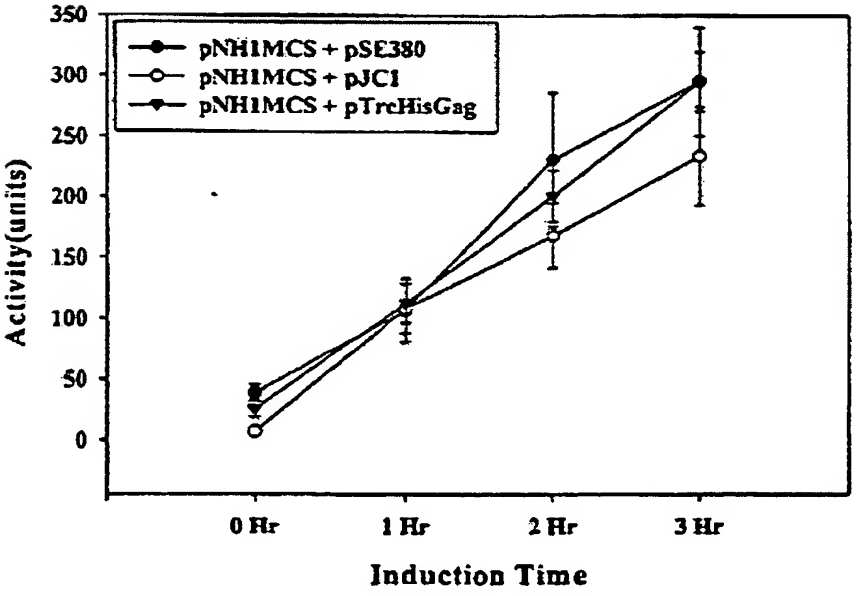
2 Hr
3 Hr
Induction Time

Fig. 2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Activity(units)
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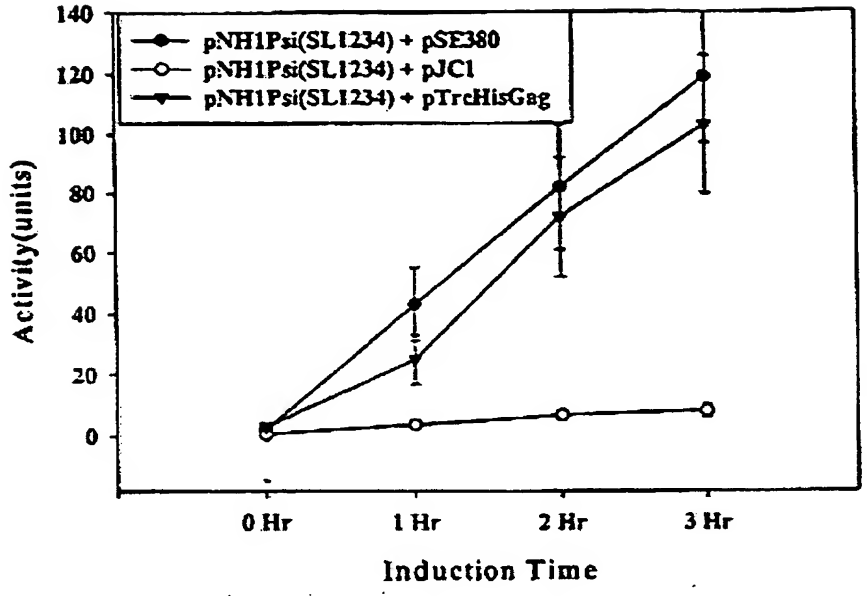
Induction Time

Fig. 3a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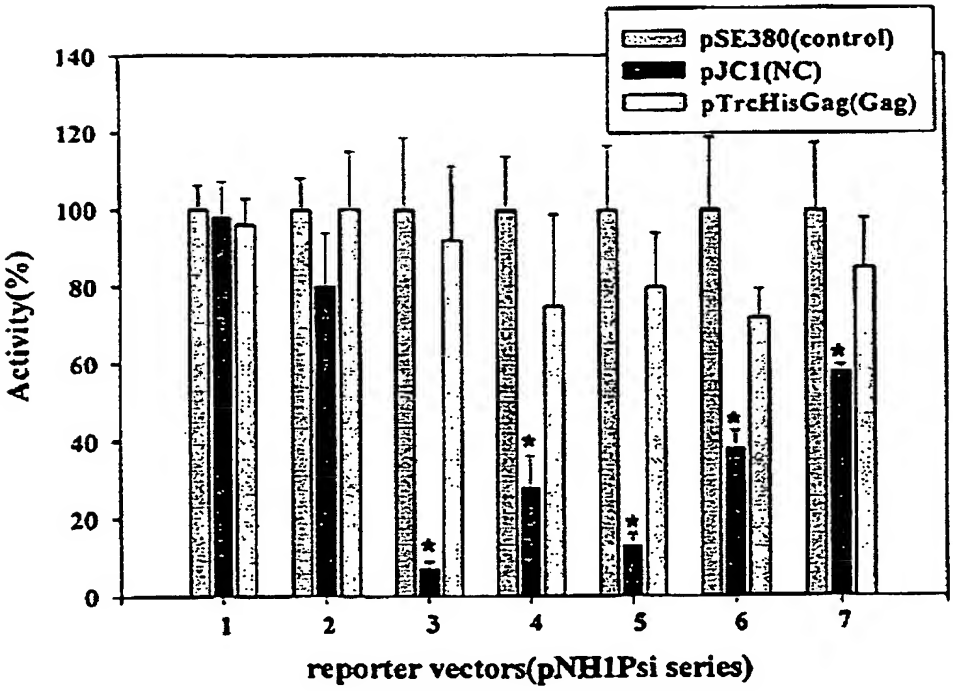
3 Hr
Induction Time

Fig. 3b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Activity(%)
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120
100
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2
3
4
5
6
7
reporter vectors(pNH1Psi series)

Fig. 4

Sequence Listing

<110> YOU, Ji-Chang

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR00/01173

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/03

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimun documentation searched (classification system followed by classification symbols)
IPC(7) C12N

Documentation searched other than minimun documentation to the extent that such documents are included in the fileds searched
Korean Patent and application for inventions since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search trerms used)
IPN, NPS, PAJ, Medline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	YOU JC et al., "HIV nucleocapsid protein. Expression in Escherichia coli, purification, and characterization." , J. Biol. Chem., August 1993 268(22), pages 16519-16527.	1
A	STAIR RK et al., "Recombinant retroviral systems for the analysis of drug resistant HIV.", Nucleic Acids Res., October 1993, 21(20), pages 4836-4842.	1, 2
A	BAUM EZ et al., "beta-Galactosidase containing a human immunodeficiency virus protease cleavage site is cleaved and inactivated by human immunodeficiency virus protease.",Proc Natl Acad Sci U S A, December 1990, 87(24), pages 10023-10027.	2

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 FEBRUARY 2001 (12.02.2001)	14 FEBRUARY 2001 (14.02.2001)

Name and mailing address of the ISA/KR	Authorized officer
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